

U.S. DEPARTMENT OF COMMERCE PATENT & TRADEMARK OFFICE

B/O Form PTO-1390 Transmittal Letter to the United States Designated/Elected Office (DO/EO/US) Concerning a Filing Under 35 USC 371		Attorney's Docket Number 1268-107
		U.S. Application Number 09/647,952
International Application Number PCT/IL99/00184	International Filing Date 30 March 1999	Priority Date Claimed April 1998
Title of Invention RECOMBINANT POTYVIRUS CONSTRUCT AND USE THEREOF		
Applicant(s) for DO/EO/US Amit GAL-ON		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items under 35 USC 371:

1. ☒ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371.
2. ☒ An executed oath or declaration of the inventor(s) (35 USC 371(c)(4)).
3. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).
4. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
5. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
6. ☐ A **FIRST** preliminary amendment.
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
7. ☐ A substitute specification.
8. ☐ A change of power of attorney and/or address letter.
9. ☒ Other items or information: A copy of PTO Form PCT/DO/EO/905 and credit card authorization for the late declaration surcharge

Applicant is entitled to small entity status under 37 C.F.R. § 1.9(f)

The fees are enclosed by way of credit card authorization. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to **Deposit Account Number 07-1337**. A duplicate copy of this sheet is enclosed.

Respectfully submitted,

Benjamin J. Hauptman
Registration Number 29,310

LOWE HAUPTMAN GILMAN & BERNER, LLP

1700 Diagonal Road
Suite 310
Alexandria, Virginia 22314
(703) 684-1111

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FORM PTO-1390

U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

1268-107

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)

09/647952

INTERNATIONAL APPLICATION NO.

PCT/IL99/00184

INTERNATIONAL FILING DATE

30 March 1999(30.03.99)

PRIORITY DATE CLAIMED

7 April 1998 (07.04.98)

TITLE OF INVENTION

RECOMBINANT POTYVIRUS CONSTRUCT AND USE THEREOF

APPLICANT(S) FOR DO/EO/US

Amit GAL-ON

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the
3. ☐ expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☒ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendment has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. Below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☒ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information.
 - a. PCT/ISA/220 – Notification of Transmittal of The International Search Report or the Declaration
 - b. PCT/IPEA/416 – Notification Of Transmittal Of International Preliminary Examination Report (Amended Claim 1; Sheet No. 26)

U.S. APPLIC. NO. (if known, see 37 CFR 1.5) 09/647952		INTERNATIONAL APPLICATION NO. CT/IL99/00184		ATTORNEY'S DOCKET NUMBER 1268-107	
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17. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1,000.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO and JPO \$ 860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$ 710.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$ 690.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) And all claims satisfied provisions of PCT Article 33(2)-(4) \$ 100.00 <div style="text-align: right;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div>					
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 0.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	20 - 20 =	0	X \$18.00	\$ 0.00	
Independent Claims	3 - 3 =	0	X \$80.00	\$ 0.00	
Multiple dependent claim(s) (if applicable)			+ \$270.00	\$ 0.00	
TOTAL OF ABOVE CALCULATIONS =				\$ 860.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$ 0.00	
SUBTOTAL =				\$ 0.00	
Processing fee of \$130.00 for furnishing the English translation later than the <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$ 0.00	
TOTAL NATIONAL FEE =				\$ 860.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				\$ 0.00	
TOTAL FEES ENCLOSED =				\$ 860.00	
				Amount to be:	\$
				refunded	
				charged	\$

a. ☒ Please charge the amount of **\$860.00** to American Express 3728 327418 95003

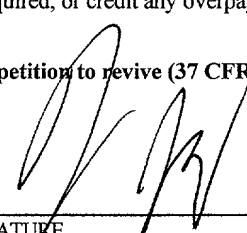
b. ☐ Please charge my Deposit Account No. XXX in the amount of \$ XXX to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 07-1337. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Benjamin J. Hauptman
LOWE HAUPTMAN GOPSTEIN GILMAN & BERNER, LLP
 1700 Diagonal Road, Suite 310
 Alexandria, VA 22314
 (703) 684-1111


 SIGNATURE
Benjamin J. Hauptman
 NAME
29,310
 REGISTRATION NUMBER

Docket No.: 1268-107

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :
Amit GAL-ON :
Serial No. Not Yet Assigned : Group Art Unit: Not Yet Assigned
Filed: October 6, 2000 : Examiner: Not Yet Assigned

For: RECOMBINANT POTYVIRUS CONSTRUCT AND USE THEREOF

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
Washington, D. C. 20231

Sir:

Preliminary to examination of the above-referenced application, please amend the application:

IN THE CLAIMS:

In claim 6, line 2, delete "-5"

In claim 8, line 1, delete "-7"

In claim 10, line 1, delete ",8 and 9"

In claim 11, line 2, delete "-6"

In claim 12, line 1, change "claims 1-11" to "claim 1"

In claim 16, line 3, change "any of the preceding claims" to "claim 1"

Serial No. Not Yet Assigned

Claim 17 (Amended) A virus containing the recombinant construct as defined by claim 1 and as produced according to [claim 16] a method for the production of a mild strain of potyvirus comprising inoculating plants with the recombinant potyvirus infectious construct and collecting the results progeny.

Claim 18 (Amended) Produce inoculated with the recombinant construct as defined in [the preceding claims] claim 1 and in the method according to [claim 13] a method for providing protection against viral infection in plants comprising inoculating plants with the recombinant potyvirus infectious construct.

Claim 20 (Amended) Compositions for plant inoculation or for transient expression of foreign nucleic acid in plants containing, as an active ingredient, the recombinant construct according to claim 1 [-12] or a virus containing the recombinant construct according to [claim 17] a virus containing the recombinant construct and as produced according to a method for the production of a mild strain of potyvirus comprising inoculating plants with the recombinant potyvirus infectious construct and collecting the results progeny.

[illegible]

REMARKS

The above-referenced application is amended to delete the multiple dependencies of claims 6, 8, 10, 11, 12, 16, 17, 18, and 20 to avoid the multiple dependent claim filing fee.

Respectfully submitted,

LOWE HAUPTMAN GOPSTEIN GILMAN & BERNER

Benjamin J. Hauptman
Registration No. 29,310

1700 Diagonal Road, Suite 310
Alexandria, Virginia 22314
(703) 684-1111
(703) 518-5499 Facsimile
Date: October 6, 2000
BJH:rl

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WO 99/51749

RECOMBINANT POTYVIRUS CONSTRUCT AND USE THEREOF

Field of the Invention

The present invention generally relates to a recombinant potyvirus infectious nucleic acid construct useful for providing protection against viral infection in plants and to a recombinant virus harboring said construct. More specifically, the present invention relates to a recombinant potyvirus infectious construct containing an HC - Pro gene whose sequence coding for the conserved FRNK box contains a substitution. Preferably, the Arginin (Arg) is substituted with Isoleucine (Ile).

The present invention further relates to a method for the production of a mild strain of potyvirus utilizing the above mentioned construct and to a method for protecting plants from viral infection and for transient expression of foreign nucleic acid (genes) in plants, using said construct. Preferably, the present invention relates to a method for cross protection of curcubits against ZYMV infection.

Background of the Invention

The Curcubitaceae is a broad botanical family comprising several economically important species cultivated worldwide, such as cucumber, squash, cantaloupe, zucchini pumpkin, melon and watermelon. Curcubit production throughout the world is impaired by several aphid transmitted viruses, the most prevalent being the two potyviruses ZYMV (Zucchini Yellow Mosaic Virus) and WMV-2 (Watermelon Mosaic Virus 2) and CMV (cucumber Mosaic Virus). ZYMV infected plants show symptoms such as vein clearing followed by a yellow mosaic on the infected systemic leaf and may show stunting and distortion. In mild cases of infection the quantity and quality of the yield are damaged and in severe infections there might be a total loss of the yield, causing significant economical losses.

Control measures include phytosanitation, the use of colored plastic mulches for attracting virus bearing aphids and creating a hydrophobic barrier around the plant such as oil sprays. These provide temporary protection and are a limited protection during a massive infection.

Development of virus resistant cultivars either by classical breeding or by introducing viral derived nucleic acid sequences into the plant genome through genetic engineering of plants, is also employed for the protection of plants against virus infection. Squash hybrid transgenic inbred lines exhibiting resistance to ZYMV were produced (Tricoli D.M., Carney K.J., Russell McMaster P.F., Groff D.W., Hadden K.C., Himmel P.T., Hubbard J. P., Boeshore M.L. and Quemada H.D. (1995) *Biotechnology* vol. 13;1458) but these are limited to one cultivar only.

The phenomenon of cross protection, which is the use of a mild strain of a virus to protect against the damage by infection with severe strains of the same virus, provides a good method for controlling virus diseases.

In cucurbits, cross protection, specifically against ZYMV, is an attractive control option. Cross protection is highly effective under severe disease pressure. The severity of the disease conferred by the ZYMV on cucurbits and the latter's relatively short crop cycle (8 - 16 weeks) make cross protection a preferred control option for cucurbits.

The currently used mild strain of ZYMV for cross protection of cucurbits, was obtained by Lecoq (Lecoq H., Lemaire J.M., Wipf-Scheible C., (1991) *Plant Dis.* 75:208-211). This strain is designated ZYMV-WK and is poorly transmitted by aphids, causes only mild leaf mottling and does not induce fruit malformation in cucurbits. Plants are inoculated at an early stage with the mild strain (ZYMV-WK), usually by mechanical inoculation.

No full length infectious clone of this mild virus exists.

Potviruses have a genome consisting of a positive - sense single stranded RNA possessing a covalently linked 5' - terminal viral protein (Vpg) and a 3' terminal poly (A) tail. The viral RNA is expressed as a single polyprotein,

which is subsequently processed by three virus encoded proteases, producing eight to ten genes, which are a conserved region throughout the potyvirus genome. The potyviruses are transmitted from plant to plant by aphids in a non persistent manner, and this process is dependent on the presence of two virus encoded proteins, the coat protein (CP) and the helper component proteinase HC-Pro. The HC-Pro is a multifunctional protein involved in aphid transmission, polyprotein processing, virus replication, symptom expression and in virus movement in the plant (Maia I. G., Haenni A., and Bernardi F., (1996) *Journal of General Virology* 77:1335-1341).

Zucchini yellow mosaic virus (ZYMV) is a member of the potyvirus group which causes devastating epidemics in commercial cucurbits world wide. A full length clone of ZYMV, from which infectious transcripts were produced, was constructed (Gal On A., Antignus Y., Rosner A., and Raccach B. (1991) *Journal of General Virology* 72:2639-2643).

It was found that a substitution of the Alanin (Ala) residue to Threonin (Thr) at position 10 in the conserved DAG (Aspartate - Alanin - Glycine; Asp-Ala-Gly) triplet in the N terminal region of the CP effectively abolished aphid transmissibility of ZYMV (Gal On A., Antignus Y., Rosner A., and Raccach B. (1992) *Journal of General Virology* 73:2183-2187). Also substitution of Thr by Ala at position 309 in the HC-Pro gene of the infectious clone of ZYMV effected aphid transmissibility without changing virus accumulation and symptom development (Huet H., Gal On A., Meiri E., Lecoq H. and Raccach B. (1994) *Journal of General Virology* 75:1407-1414), though less effectively than the substitution in the DAG triplet in the CP of the ZYMV.

It has surprisingly been found that an amino acid substitution in the conserved FRNK box of the potyvirus HC-pro gene allows for the construction of an infectious potyvirus construct, which, when introduced to plants, induces little or no symptom development, and which does not effect the accumulation of the virus in the plant. This infectious construct is therefore a unique potyvirus construct which is highly superior for plant cross protection and for transient

expression of foreign nucleic acid in plants. It has an improved ability of protection against infection by the severe strain of ZYMV, over any of the existing protection methods, is significantly safer and more environment friendly than the naturally occurring viruses, does not cause the development of symptoms in a variety of cucurbits, and is stable (no revertant virus has been found after several passages through plants).

Summary of the Invention

The present invention relates to a recombinant potyvirus infectious nucleic acid, preferably cDNA or RNA, construct useful for plant cross protection, comprising a full length clone characterized in that its HC- Pro gene conserved FRNK box sequence contains a substitution, preferably a substitution of Arg.

Preferably, the construct further contains a substitution which effectively abolishes aphid transmissibility, such as a substitution of the Ala residue at position 10 in the conserved DAG triplet in the N terminal region of the CP or substitution of Thr at position 309 in the HC - pro in ZYMV.

The recombinant construct of the present invention may be useful for plant cross -protection (especially against severe strains of ZYMV) and for the transient expression of foreign nucleic acid in plants wherein the full length clone has, in any position, a sequence of DNA or RNA inserted into it or wherein any viral genes are removed from the full length clone (defective RNA). The full length clone may be of any potyvirus, preferably of ZYMV.

The present invention also relates to a method for providing protection against viral infection in plants comprising inoculating plants, by mechanical inoculation or by bombardment, with the recombinant potyvirus infectious construct.

The present invention also relates to a method for introducing foreign nucleic acid into plants according comprising infecting a plant with a full length clone or co-infecting a plant with a full length clone, from which any viral genes are

removed, together with a full length clone or virus harboring a full length clone.

The present invention also relates to a method for the production of a mild strain of potyvirus comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims and collecting the resulting progeny, and to a virus produced in this method.

The present invention further relates to compositions for plant inoculation or for transient expression of foreign nucleic acid in plants containing, as an active ingredient, the recombinant construct or a virus containing the recombinant construct.

Detailed Description of the Invention

The present invention relates to a recombinant potyvirus infectious nucleic acid construct useful for plant cross protection and for the transient expression of foreign nucleic acid in plants. The present invention further relates to compositions for plant inoculation or for transient expression of foreign nucleic acid in plants containing, as an active ingredient, the recombinant construct or a virus containing the recombinant construct.

The construct of the present invention comprises a full length potyvirus clone containing a substitution in the conserved FRNK box sequence in the HC - pro gene, preferably, Arg (in the FRNK box) is substituted with an amino acid having a bulky side chain or an amino acid from the hydrophobic group such as Ile. This substitution in the FRNK box dramatically effects the severity of symptom development without effecting the accumulation of the virus in the plant. Preferably, the construct of the present invention also contains a substitution which effectively abolishes aphid transmissibility, such as the substitution of the Ala residue to Thr at position 10 in the conserved DAG (Asp-Ala-Gly) triplet in the N terminal region of the CP or substitution of Thr by Ala at position 309 in the HC - pro of ZYMV.

Full length infectious clones of a severe strain of ZYMV were constructed and put under the control of a phage promoter, such as the T7 RNA polymerase promoter (Gal On A., Antignus Y., Rosner A., and Raccach B. (1991) *Journal of General Virology* 72:2639-2643), bacterial promoters or a promoter effective *in planta*, such as the cauliflower mosaic virus (CaMV) 35S promoter (Gal On A., Meiri E., Huet H., Hua W.J., Raccach B. and Gaba V. (1995) *Journal of General Virology* 76:3223-3227).

In the work presented here, the FRNK box is implicated, for the first time, as being of importance in symptom development, surprisingly without effecting the accumulation of the virus in the plant. Due to the highly conserved sequence of the FRNK box within the HC -Pro gene of the potyviruses, any substitution in the FRNK box of a potyvirus would have an effect on symptom development, not only the substitution of Arg in position 180 with Ile, in ZYMV, demonstrated in the work described here.

Based on the highly conserved genome, organization and gene function of the potyviruses, it may be concluded that the conserved FRNK box in the HC - pro gene has the same function in all potyviruses (perhaps as a receptor). Therefore, the substitution in the FRNK box in any of the potyviruses would have a similar effect on symptom development. Members of the potyviruses that are economically important are, for example, BCMV (Bean Common Mosaic Virus), BYMV (Bean Yellow Mosaic Virus), BtMV (Beet mosaic), MWMV (Moroccan watermelon mosaic), OYDV (Onion yellow dwarf), PRSV (Papaya ringspot), PStV (Peanut stripe), PepMoV (Pepper mottle), PVMV (pepper veinal mottle), CGVBV (Cowpea green vein banding), GEV (ground eyespot), ISMV (Iris severe mosaic), JGMV (Johnsongrass mosaic), LYSV (Leek yellow stripe), LMV (Lettuce mosaic), MDMV (Maize dwarf mosaic), PPV (Plum box), PVA (Potato A), PVV (Potato V), PVY (Potato Y), SbMV (Soybean mosaic), SCMV (Sugarcane mosaic), SPFMV (Sweet potato feathery mottle), TEV (Tobacco etch), TVMV (Tobacco vein mottling), TBV (Tulip

breaking), TuMV (Turnip mosaic), WMV-2 (Watermelon Mosaic Virus 2) , YMV (Yam mosaic), ZYFV (Zucchini yellow fleck).

The infectious clone may be an RNA transcript or a cDNA construct, though the use of infectious transcripts is the less efficient process *in vitro*.

A method for providing protection against viral infection in plants, according to the present invention comprises inoculating plants with the recombinant potyvirus infectious construct, for example, by mechanical inoculation or by bombardment.

Compositions containing, as an active ingredient, the construct of the present invention may be used for superior plant cross protection, especially against infection by the severe strain of ZYMV and for transient expression of foreign nucleic acid in plants. The composition used for the introduction of the construct into plants, for infecting them by bombardment is an aqueous composition comprising, in approximately equal volumes, the construct, a salt, such as calcium nitrate and particles such as tungsten, gold. The composition used for the introduction of the construct into plants by mechanical inoculation comprises infected plant tissue.

The construct of the present invention may be further used as a vehicle for the transient expression of foreign nucleic acid, namely genes, in a plant. The construct according to the present invention is highly infective, does not induce symptoms in the infected plants and is not transmitted by aphids.

Use of compositions, containing as an active ingredient, this clone provides an efficient, safe and environment friendly method for transient expression of foreign nucleic acid into the infected plants. Further applications of this construct may, therefore, be the expression of foreign sequences or genes within a defective RNA molecule of potyviruses. Defective RNAs are viral RNA genomes which are missing some of the viral genes but which, together with a complete helper virus (the full length parental virus), can facilitate the expression of the sequences they contain. Defective RNAs are derived from the helper virus genome, but still require the presence of a complete helper

virus for replication in the plant cell. The construct of the present invention may have viral genes removed from the full length clone and may then serve to support the expression of foreign genes via potyviruses defective RNA by co-infection of a plant with a full length clone, from which any viral genes are removed, together with a full length clone or virus harboring a full length clone.

A method for introducing foreign nucleic acid into plants according to the present invention comprises infecting a plant with a full length clone into which any sequence of DNA or RNA is inserted or co-infecting a plant with a full length clone, from which any viral genes are removed, together with a full length clone or virus harboring a full length clone.

A method for the production of a mild strain of potyvirus, according to the present invention comprises inoculating plants with the recombinant potyvirus infectious construct and collecting the resulting progeny.

The said invention will be further described and illustrated by the following experiments and figure. These experiments and figure do not intend to limit the scope of the invention but to demonstrate and clarify it only.

Brief Description of the Figure

Figure 1 is a schematic drawing of four constructs of ZYMV infectious cDNA (a-d).

Detailed Description of the Figure

Figure 1 is a schematic drawing of four constructs of ZYMV infectious cDNA (a-d). The open and striped bars indicate the ZYMV-NAT and ZYMV-WK sequences within the FLC respectively. The relevant restriction enzymes and the amino acid changes are present. On the right side the severity of the symptoms in squash is indicated, from very severe (+++++) to mild (+). The sequence of the primer used for the mutagenesis is indicated.

Example 1 - full length clone (FLC) of ZYMV

Construction of the mutants in the full length clone (FLC) of ZYMV

The constructs which represent the HC - Pro sequences (Huet H., Gal On A., Meiri E., Lecoq H. and Raccach B.(1994) *Journal of General Virology* 75:1407-1414) of the ZYMV - WK strain were placed under the T7 RNA promoter in the infectious FLC. In order to get higher rate of infection with those constructs the fragment BstXI/AgeI from the FLC of 35SZYMVNOS cDNA (Gal On A., Antignus Y., Rosner A., and Raccach B. (1991) *Journal of General Virology* 72:2639-2643 and Gal On A., Meiri E., Huet H., Hua W.J., Raccach B. and Gaba V. (1995) *Journal of General Virology* 76:3223-3227), was replaced by the appropriate fragment from pZYHC (-) clone (Huet H., Gal On A., Meiri E., Lecoq H. and Raccach B.(1994) *Journal of General Virology* 75:1407-1414). Site directed mutagenesis was introduced on ssDNA template of the subclone pksM16B (Gal On A., Antignus Y., Rosner A., and Raccach B. (1991) *Journal of General Virology* 72:2639-2643), using the primer 5' ATGTTCATAAATAAGCGCTCTAG3' (amino acid Ile is underlined and the unique restriction site of Eco47III is in bold). The clone pksM16B carrying the mutations was double digested by BamHI/BstEII and the obtained fragment (1.4kb) was introduced to the same sites in the 35SZYMVNOS cDNA (Gal On A., Meiri E., Huet H., Hua W.J., Raccach B. and Gaba V. (1995) *Journal of General Virology* 76:3223-3227).

Plants, mechanical or bombardment inoculation and symptom appearance of the ZYMV AG1

Greenhouse - grown zucchini squash (*Curcubita pepo*. L. cv Ma'ayan), cucumber (*Cucumis sativus* L. cv. Bet Alpha; Shimshon; Delila), melon (*Cucumis melo* L. cv. Arava) and watermelon (*Citrullus lanatus* Schad cv. Malali) plants were used at the cotyledon stage. The inoculated plants were maintained in a growth chamber under continuous light at about 25°C. The plants were examined daily for visual symptom development.

Bombardment inoculation were as previously described by Gal - On et al. (1995). Mechanical inoculation of plants infected by the recombinant virus were performed by sap inoculation (100mg/ml), applied to a cotyledon previously dusted with carborundum.

Cross protection experiments

Cross protection by the ZYMV-AG1 strain was tested as described by Lecoq et al. (1991) Squash seedlings at the fully expanded cotyledon stage were bombarded with the 35S-AG1 at 0.1 µg/µl. A week later they were infected in the greenhouse by 5 - 7 aphids (*Myzus persicae*) per plant according to Antignus Y., Raccach B., Gal - On A. and Cohen S. (1989) *Phytoparasitica* 17:287-289).

Determination of the mutation in the progeny virions

To ascertain the presence of the mutations in the viral RNA total mRNA from infected leaf tissue was extracted. The synthesis of the RT-PCR was performed as described by Huet et al. (1994).

ELISA assay for evaluation of ZYMV titer

Leaf discs of squash and cucumber ZYMV-infected plants were taken 7 - 10 d.p.i. and the homogenized tissue were subjected to ELISA as described by Antignus et al (1989).

Previously, sequence comparison has shown four amino acid changes in the 455 amino acid sequence of the HC - pro gene between the severe field strain (ZYMV - JV) and the mild field strain ZYMK - WK. The replacement of a fragment of the HC - Pro of ZYMV - WK containing two substitutions Aspartate (Asp) 148 and Arg 180 (BstXI/BstEII fragment), reduced symptom expression of the virus in squash plants without effecting virus accumulation. To distinguish which of the two substitutions, Asp 148 or Arg 180, effect

symptom development, Arg 180 was replaced by Ile within the FRNK box (figure 1, clone d) by site directed mutagenesis.

The engineered virus containing the Arg 180 replacement by Ile, was designated ZYMV-AG1. This new strain did not cause the development of symptoms in cucumber (three different varieties), melon and watermelon. The virus did accumulate to levels as high as that of the wild type ZYMV-JV. It was assumed, therefore, that the second amino acid difference (Asp at position 148) is dispensable for altering the symptoms from mild to severe.

In order to verify the presence of the amino acid changes within the mild virus ZYMV - AG1, and to prevent aphid transmission, a new restriction site of Eco47III was introduced at position 550 nt (from the 5' of the HC- Pro gene) and the DAG motif in the CP was replaced by DTG respectively (figure 1).

The new engineered virus (AG1) and a wild type severe strain (JV) accumulated to a similar level in systemically infected leaves of different curcubit species (Table 1). Therefore, it may be concluded, that a point mutation changing amino acid Arg 180 to Ile, dramatically effects the severity of symptom development without effecting the movement and the replication of the ZYMV virus in the plant. The dramatic results conferred by a point mutation in the potyvirus FRNK box, demonstrated in this work for the first time, could not have been inferred from the mere known sequence comparison which showed amino acid changes between the severe field strain and the mild field strain.

The stability of the amino acid substitution Arg 180 to Ile within ZYMV-AG1 was tested by infecting hundreds of squash plants and dozens of cucumber plants (Table 2). The presence of the Ile 180 mutation in the HC - Pro was confirmed by sequencing (data not shown). Curcubit plants inoculated with ZYMV-AG1 mechanically or by particle bombardment with the ZYMV-AG1 strain did show the mild symptom appearance even throughout the growing period of the plant (Table 2). The presence of the Ile 180 mutation within the

virion genome was confirmed by sequencing or indirectly by digestion of the RT-PCR amplified fragment with the restriction enzyme Eco47III (figure 1).

Replication and movement of the engineered ZYMV-AG1 strain remained high (as the wild type ZYMV), as seen from the accumulated level of the virus. These results suggest that no selective pressure is exerted to cause a reversion in the virus mutated genome.

The ability of the newly produced mild strain (ZYMV-AG1) to protect against a challenge inoculation of the severe strain of ZYMV (JV), was studied in cross protection experiments. Most of the protected plants did show mild symptoms after a challenge with the severe strain (96% protection). Two plants out of 47 that were infected with the ZYMV-AG1 strain and challenged a week later with the JV strain exhibited severe symptoms about one month post inoculation (Table 3).

The protection was studied in a small field experiment in which protected plants were exposed to field inoculation. Approximately 40% of the control non-protected plants became infected, while none of the protected plants showed severe symptoms. Therefore, no fruit damage was observed in the protected plants (Table 3). Previous studies showed that in a typical cross protection phenomenon, both the protective and the challenge virus strains are very closely related (Perring T.M., Farrar C. A., Blua M. J., Wang H.L. and Gonsalves D. (1995) *Crop Protection* 14 no. 7, 601 - 606). This is the first report where cross protection takes place between strains that have an identical sequence, including the coat protein sequence, that differ only in a single amino acid in a non structural protein (the HC - Pro).

2) Cross protection in melons

Melon (*Cucumis melo* L. cv. Ofir) seedlings were planted and were infected with ZYMV-WK and the recombinant virus ZYMV-AG1. The viruses were sprayed onto the melon seedlings prior to planting. The seedlings were then planted together with untreated (control) seedlings.

Half of the plants at three weeks were challenged mechanically with the wild type virus (ZYMV-JV) and half were unchallenged for testing natural infection.

30 days after the beginning of the experiment parameters such as the plant size and the extent of infection with the wild type virus, were studied. Plants infected with ZYMV-JV that were not treated by the weakened virus (WK) were small and showed clear infection symptoms. Plants treated with the recombinant virus (ZYMV - AG1) showed no symptoms of infection.

3) Expression of foreign genes through the ZYMV-AG1 clone in plants
For the expression of a foreign gene in an infected plant, a Pst I site was inserted into the ZYMV-AG1 between the N1b and CP genes. The GFP (green fluorescent protein) reporter gene and the Bar gene, which confers resistance to the non selective herbicide bialaphos (commercially named BASTA), were amplified by PCR, using primers containing the Pst I restriction site, and were inserted in the PstI site.

Plants were inoculated by bombardment with the ZYMV - AG1 containing the GFP reporter gene or Bar gene.

Biochemical analysis showed the GFP and Bar gene to be highly and stably expressed. Even after several passages, no revertants of the recombinant mild virus were found and the reporter gene and Bar expression remained high and stable. Plants expressing the GFP were luminescent and plants expressing the Bar gene were found resistant to the herbicide bialaphos.

Table 1. Comparison of virus accumulation between ZYMV-JV and ZYMV-AG1 strains in cucurbits.

experiment no.	no of tested plants: JV, AGI, WK	ZYMV-JV# severe ELISA OD(405)	ZYMV-AG1^ mild	ZYMV-WK~ mild
1s+	11, 6, 6	0.9* (0.41)**	0.5 (0.19)	0.7 (0.18)
2s	2, 9, 8	1 (0.4)	0.7 (0.48)	-
3s	3, 10, 4	0.3 (0.08)	0.9 (0.27)	1.33 (0.13)
4s	9, 9, 9	0.51 (0.4)	0.46 (0.21)	0.59 (0.3)
5s	9, 9, -	0.56 (0.07)	0.7 (0.09)	-
6s	9, 8, -	0.82 (0.09)	0.95 (0.09)	-
7c	6, 7, -	0.7 (0.07)	0.81 (0.2)	-

Severe strain of ZYMV which found in Israel in the Jordan Valley (JV).

^ The engineered virus of ZYMV.

~ ZYMV weak strain described by Lecoq et al. (1991).

* Average of O.D (405) detected by ELISA from 11 plants.

** Standard deviation (in brackets).

+ s and c are squash and cucumber test plants, respectively.

Table 2. The stability of the ZYMV-AGI virus in the plants

plant species	bombardment with 35SAG1	<u>number of tested plants</u>		
		* visual		# molecular
		symptoms		analysis of Ilu-180
		mild	severe	mutation
squash	402	398	0	10
cucumber	105	103	0	5
melon	30	30	0	3
Total	537 [^]	531 ⁺	0	18

*Visual symptoms were observed and detected by ELISA, about one and half month post inoculation.

The presence of the Ilu Mutation was confirmed by digestion of the RT-PCR by Eco47III restriction enzyme.

[^] Total of bombarded plants.

⁺ Total of infected plants

Table 3. Cross protection in squash with the mild strain ZYMV-AG1 (induction) against the severe strain ZYMV-JV (challenge) in the greenhouse experiments.

experiment number*	induction ZYMV-AG1	<u>Number of tested plants</u>		~fruit damage
		#challenge ZYMV-JV	symptoms mild severe	
a)	47	47	45 2	1
a)	14	-	15	0
a)	-	5	5	5
b)	15	15	14 0	0
b)	5	-	5	0
b)	-	5	5	5
c)	43	field inocul.	43	0
c)	-	6	6	6
c)18 healthy	-	field inocul.	7	7

* a, b and c are three separate experiments. a and b were in the greenhouse and c was done in a small plot in the field. c is a sum of two experiments where the protected plants (AGI) were exposed to field inoculation.

~ No. of plants showed fruit damage.

Inoculation by aphids.

CLAIMS

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- 1) A recombinant potyvirus infectious nucleic acid construct useful for plant cross protection, comprising a full length clone characterized only in that its HC-Pro gene conserved FRNK box sequence contains a substitution.
- 2) A recombinant construct according to claim 1 wherein the nucleic acid is cDNA or an RNA transcript.
- 3) A recombinant construct according to claim 1 wherein the substitution in the conserved FRNK box is a substitution of Arg.
- 4) A recombinant construct according to claim 3 wherein Arg is substituted with an amino acid of the hydrophobic group or having a bulky side chain.
- 5) A recombinant construct according to claim 4 wherein Arg is substituted with Ile.
- 6) A recombinant potyvirus infectious nucleic acid construct according to claim 1-5 wherein the potyvirus is ZYMV.
- 7) A recombinant construct according to claim 6 wherein the construct is ZYMV-AG1.
- 8) A recombinant construct according to claim 1-7 further containing a substitution which effectively abolishes aphid transmissibility.
- 9) A recombinant construct according to claim 8 wherein the substitution which effectively abolishes aphid transmissibility is a substitution of the Ala residue at position 10 in the conserved DAG triplet in the N terminal region of the CP.

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10) A recombinant construct according to claim 7,8 and 9 useful for plant cross protection wherein the cross protection is against severe strains of ZYMV.

11) A recombinant potyvirus infectious nucleic acid construct according to claims 1-6 wherein the potyvirus is selected from BCMV, BYMV, BtMV, MWMV, OYDV, PRSV, PStV, PepMoV, PVMV, CGVBV, GEV, ISMV, JGMV, LYSV, LMV, MDMV, PPV, PVA, PVV, PVY, SCMV, SPFMV, TEV, TVMV, TBV, TuMV, WMV-2, YMV and ZYFV.

12) A recombinant construct according to claims 1-11 further useful for the transient expression of foreign nucleic acid in plants wherein the full length clone has, in any position, a sequence of DNA or RNA inserted into the full length clone.

13) A method for providing protection against viral infection in plants comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims.

14) A method according to claim 13 wherein inoculating plants is done by mechanical inoculation or by bombardment.

15) A method for introducing foreign nucleic acid into plants comprising infecting a plant with a full length clone as defined in claim 11.

16) A method for the production of a mild strain of potyvirus comprising inoculating plants with the recombinant potyvirus infectious construct as defined in any of the preceding claims and collecting the resulting progeny.

17) A virus containing the recombinant construct as defined by claim 1 and as produced according to claim 16.

18) Produce inoculated with the recombinant construct as defined in the preceding claims and in the method according to claim 13.

19) Produce according to claim 18 wherein the produce are cucurbits.

20) Compositions for plant inoculation or for transient expression of foreign nucleic acid in plants containing, as an active ingredient, the recombinant construct according to claim 1-12 or a virus containing the recombinant construct according to claim 17.

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SEVERITY

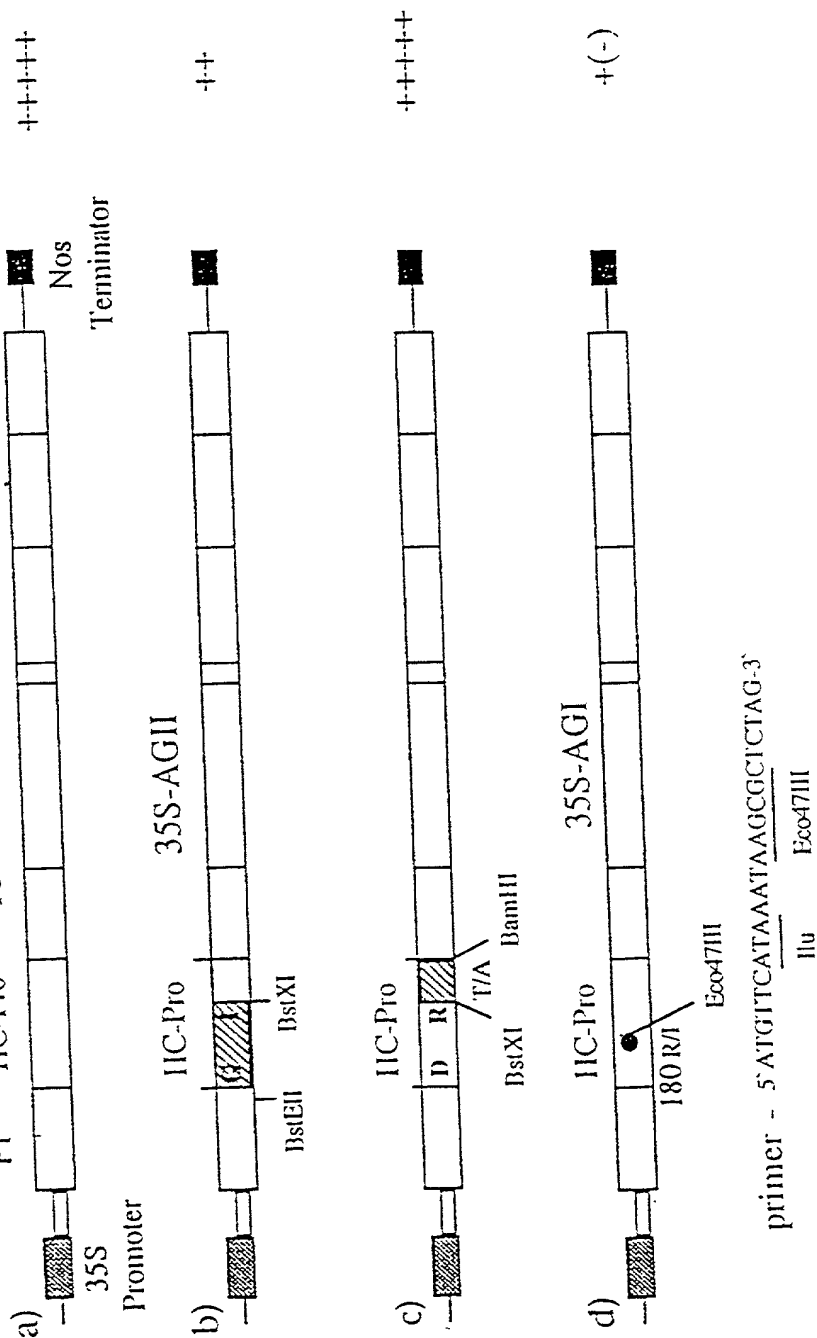


FIGURE 1

DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY

As a below-named inventor, I hereby declare that my residence, past office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention (Design, if applicable) entitled **RECOMBINANT PORCINE CONSTRUCT AND USE THEREOF** the specification of which (check one):

☐ is attached hereto.

☒ was filed on 6 October 2000, as Application No. 09/647,952, and was amended on _____ (if applicable).

☒ was filed on 30 March 1999 as International Application (PCT) No. PCT/IL99/00184, and was amended on June 1, 2000.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which the priority is claimed.

PRIORITY FOREIGN APPLICATION(S)

NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
133994	Israel	7 April 1998	<input checked="" type="checkbox"/> LI <input type="checkbox"/> CH
WO 99/82749	Wipo	30 March 1999	<input checked="" type="checkbox"/> CH <input type="checkbox"/> LI

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT International application(s) designating the United States of America (listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

APPLICATION NUMBER	FILING DATE	STATUS (Pending, Pending or Abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

POWER OF ATTORNEY: I (We) hereby appoint as my (our) attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Allan M. Lowe, Registration Number 19,641; Benjamin J. Hausman, Registration Number 29,310; Michael G. Gilman, Registration Number 19,114; Kenneth M. Berger, Registration Number 97,093; and Randy A. Normbrock, Registration Number 42,940.

Send correspondence to:


**LOWE HAUPTMAN GILMAN
& BERGER, LLP**
1700 Diagonal Road, Suite 310
Alexandria, Virginia 22314

TELEPHONE CALLS TO:

Benjamin J. Hausman
(703) 684-1111

I hereby authorize the U.S. attorneys and agents named herein to accept and following instructions from Dr. Meir Noam as to any actions to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and the undersigned. In the event of a change in the person(s) from whom instructions may be taken, the U.S. attorneys will be so notified by the undersigned.

☒ See following pages for additional joint inventors.

Full Name of Inventor or Sole Applicant AMIR GAL-ON		Citizenship Israel	
Residence Address - Street Vilna Street 16		Post Office Address Street Vilna Street 16	
City Ramat Hasharon		City Ramat Hasharon	
State or Country Israel	Zip 47295	State or Country Israel	Zip 47295
Date December 03, 2000		Signature 	

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